St. Jude Children's Research Hospital
ALSAC • Danny Thomas, Founder

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PI: Jian Zuo, Ph.D.
Organization: St. Jude Children's Research Hospital
ONR Award Number: N00014-09-1-1014
Award Title: Hearing Restoration in Mouse Models with Noise-induced Hearing Loss

Please find enclosed the Annual Technical Report of Dr. Jian Zuo. Should you have any questions or concerns, feel free to contact me at (901)595-2729.

Thanks,

Marquetta Nebo
Grant and Contract Administrator

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14. ABSTRACT
To characterize the regenerative capacity of SCs in mice after noise-induced damage and transient or permanent inactivation of p16Ink4a and assess the ability of Atoh1 to transdifferentiate SCs into HCs after noise-induced damage in mice.

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7.6.2012

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A. Scientific and Technical Objectives

As a long-term goal, we aim to develop therapeutics which would restore hearing in Navy servicemen who are suffering from noise-induced hearing loss (NIHL). Our **central hypothesis** is that inactivation of $p16^{Ink4a}$ or other cell cycle inhibitors in mammalian supporting cells (SCs) will allow them to respond to acoustically damaged hair cells (HCs) and to reenter the cell cycle; subsequently the introduction of Atoh1 will cause the newly created SCs to transdifferentiate into HCs. To test this hypothesis, we propose to develop mouse models and small molecule inhibitors to: **Aim 1**) characterize the regenerative capacity of SCs in mice after noise-induced HC damage and transient or permanent inactivation of $p16^{Ink4a}$; **Aim 2**) assess the ability of Atoh1 to transdifferentiate SCs into HCs after noise-induced HC damage in mice. Our studies will provide a basis for future clinical trials using cell cycle inhibitory and HC-differentiation promoting drugs to restore hearing in humans. Since the award, we have modified **Aim 1** to include screening of $p27^{Kip1}$ inhibitors; this is based on our recent exciting results that neonatal SCs proliferate after acute inactivation of $p27^{Kip1}$.

B. Approach

We plan to develop mouse genetic models and small molecule inhibitors to achieve the following proposed aims:

Aim 1A: To determine the regenerative capacity of SCs in $p16^{Ink4a}$-null mice after noise-induced damage.

Aim 1B: To transiently inactivate $p16^{Ink4a}$ after noise-induced damage.

Aim 1C: To develop small molecule inhibitors of $p16^{Ink4a}$ and $p27^{Kip1}$.

Aim 2A: To create and characterize transgenic mice with inducible overexpression of Atoh1 in postnatal and adult SCs and measure the effects of Atoh1 overexpression after noise-induced HC damage.

Aim 2B: To test the ability of γ-secretase inhibitors to increase Atoh1 expression in the mouse cochlea and measure their effects on HC morphology and hearing before and after noise-induced HC damage.

C. Concise Accomplishments

We have discovered *in vivo* that:

1. Noise-induced HC damage causes an increase in proliferating cells in the organ of Corti of adult $p16^{Ink4a}$ knockout mice.
2. For the first time and in contrast to common belief, the postnatal mammalian cochlea has the capacity to regenerate auditory HCs after damage, similar to non-mammalian auditory sensory epithelia. This capacity declines with age.

3. A new mouse strain, Sox10-rtTA, can be used to induce transient gene expression in SCs in neonatal and postnatal cochleae.
4. Sox2 directly regulates $p27^{Kip1}$ to maintain quiescence of postmitotic cochlear SCs.
5. Chemical compounds identified in our primary screen have the potential to inhibit $p27^{Kip1}$, a key molecule that controls proliferation of SCs.
6. SCs in the mammalian cochlea can be converted into sensory HCs in mouse models with a single factor, Atoh1.
7. Activation of Notch1 signaling can induce ectopic sensory HCs in an age–dependent manner.

Our exciting findings will allow us to further decipher the mechanisms and to develop small molecule inhibitors for restoration of hearing in Navy servicemen suffering from NIHL.

D. Expanded Accomplishments

Aim 1A: To determine the regenerative capacity of SCs in $p16^{Ink4a}$-null mice after noise-induced HC damage.

Similar to other tissues, we found that $p16^{Ink4a}$ expression is low in the cochlea of young animals and increases with age. We also found that $p16^{Ink4a}$-null mice have normal hearing and HC morphology at 1, 2 and 3 months of age. We have successfully damaged OHCs, but not IHCs, with noise exposure in adult mice and

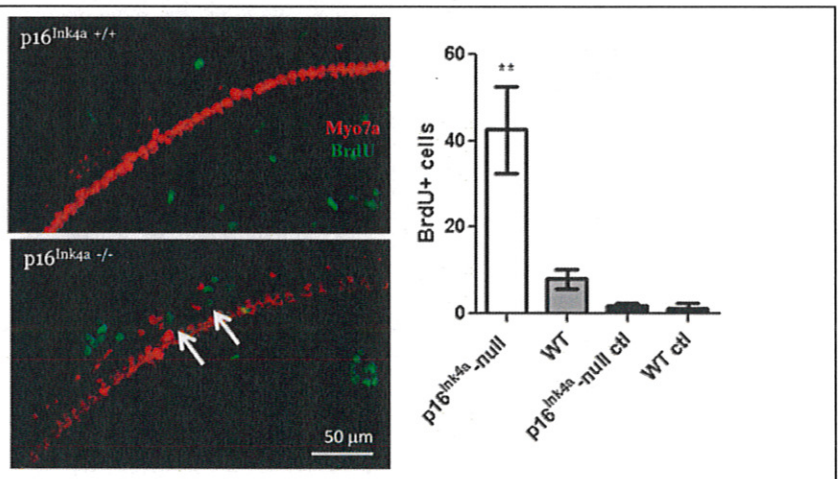


Fig. 1. Proliferating cells in the $p16^{Ink4a}$-null cochlea after n* *$p < 0.01$ compared to all other samples as determined by a one-way ANOVA followed by a Student's t test with a Bonferroni correction.

for the first time, detected a significant increase in BrdU+ cells within the organ of Corti of $p16^{Ink4a}$-null mice after noise-induced HC damage (**Fig. 1**). Interestingly, most BrdU+ cells are close to IHCs and thus could be inner pillar cells or inner phalangeal cells. Also these BrdU+ cells are not immune cells because they do not co-label with the immune cell marker, CD45. We will confirm that these BrdU+ cells are indeed proliferating SCs with definitive lineage tracing experiments. These results provide the first evidence that $p16^{Ink4a}$ plays a critical role in mammalian HC regeneration after NIHL (Cox et al., in preparation).

Non-mammalian vertebrates such as birds, fish and amphibians can regenerate HCs after damage. In contrast, damage to auditory HCs, caused by noise exposure or other factors, is currently believed to be permanent in humans and other mammals. However, the absence of HC regeneration in mammals has only been confirmed in adults. We have recently developed a novel method to damage HCs in the neonatal, mouse cochlea *in vivo* and observed spontaneous HC regeneration in the cochlea. Regenerated HCs are similar to endogenous HCs expressing four HC markers, including prestin, a terminal differentiation marker specific to outer HCs. By lineage tracing, we discovered a new mechanism of HC regeneration where SCs transdifferentiate into HCs and then proliferate (**Fig. 2**). We also defined the critical period when cochlear HC regeneration can occur (Cox et al., *Neuron* in revision).

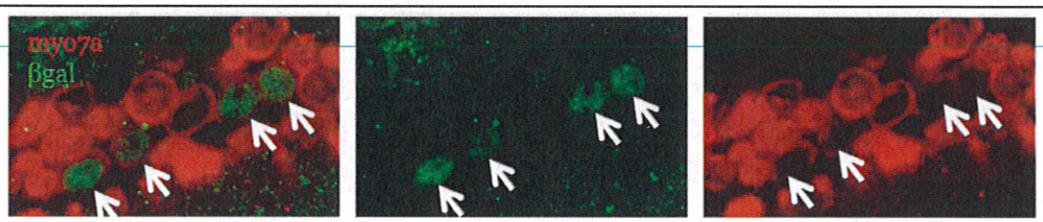


Fig. 2. Evidence of HC regeneration by transdifferentiation of SCs. Atoh1-CreERTM;Rosa26DTA/+;Hes5-nlacZ+ mice were induced at P0-1 with tomaxifen and analyzed at P2. An apical turn cochlear segment is stained with myo7a (red) and βgal (green). Arrows label Hes5-nlacZ+ and myo7a+ regenerated HCs.

Aim 1B: To transiently inactivate $p16^{Ink4a}$ after noise-induced HC damage.

We have successfully created p27-IRES-rtTA knockin mice. When crossed with two reporter lines (Tet-on-LacZ and Tet-on-Cre; Rosa26-YFP), we did not observe any reporter activity in the cochlea or cerebellum after induction with various doses of Doxycyclin in late embryonic and postnatal mice. Even after removing the neo cassette from the p27-IRES-rtTA locus and breeding homozygotes of p27-IRES-rtTA to enhance the level of rtTA, we failed to observe reporter expression in the cochlea or cerebellum. Given that the reporter worked as expected with another rtTA line, we believe that in the p27-IRES-rtTA knockin mice, the rtTA level is not high enough because the p27 level is generally low in postnatal SCs. Instead, we have characterized Sox10-rtTA mice which labels SCs at neonatal and postnatal ages (**Fig. 3**). We have thus developed an

alternative rtTA mouse line that will be extremely useful for HC regeneration studies. We believe that the phenotypes in $p16^{Ink4a}$-null mice after NIHL need further characterization, and we are therefore putting the studies of transient inactivation of $p16^{Ink4a}$ on hold.

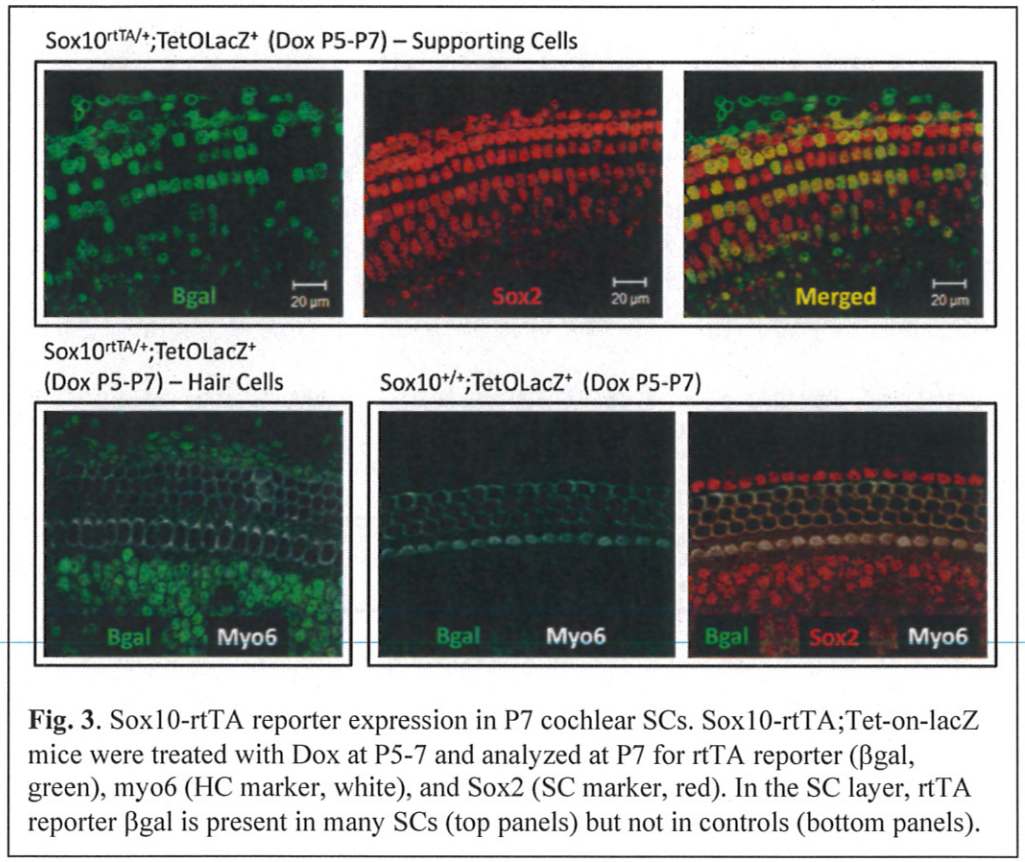


Fig. 3. Sox10-rtTA reporter expression in P7 cochlear SCs. Sox10-rtTA;Tet-on-lacZ mice were treated with Dox at P5-7 and analyzed at P7 for rtTA reporter (βgal, green), myo6 (HC marker, white), and Sox2 (SC marker, red). In the SC layer, rtTA reporter βgal is present in many SCs (top panels) but not in controls (bottom panels).

Aim 1C: To develop small molecule inhibitors of $p27^{Kip1}$.

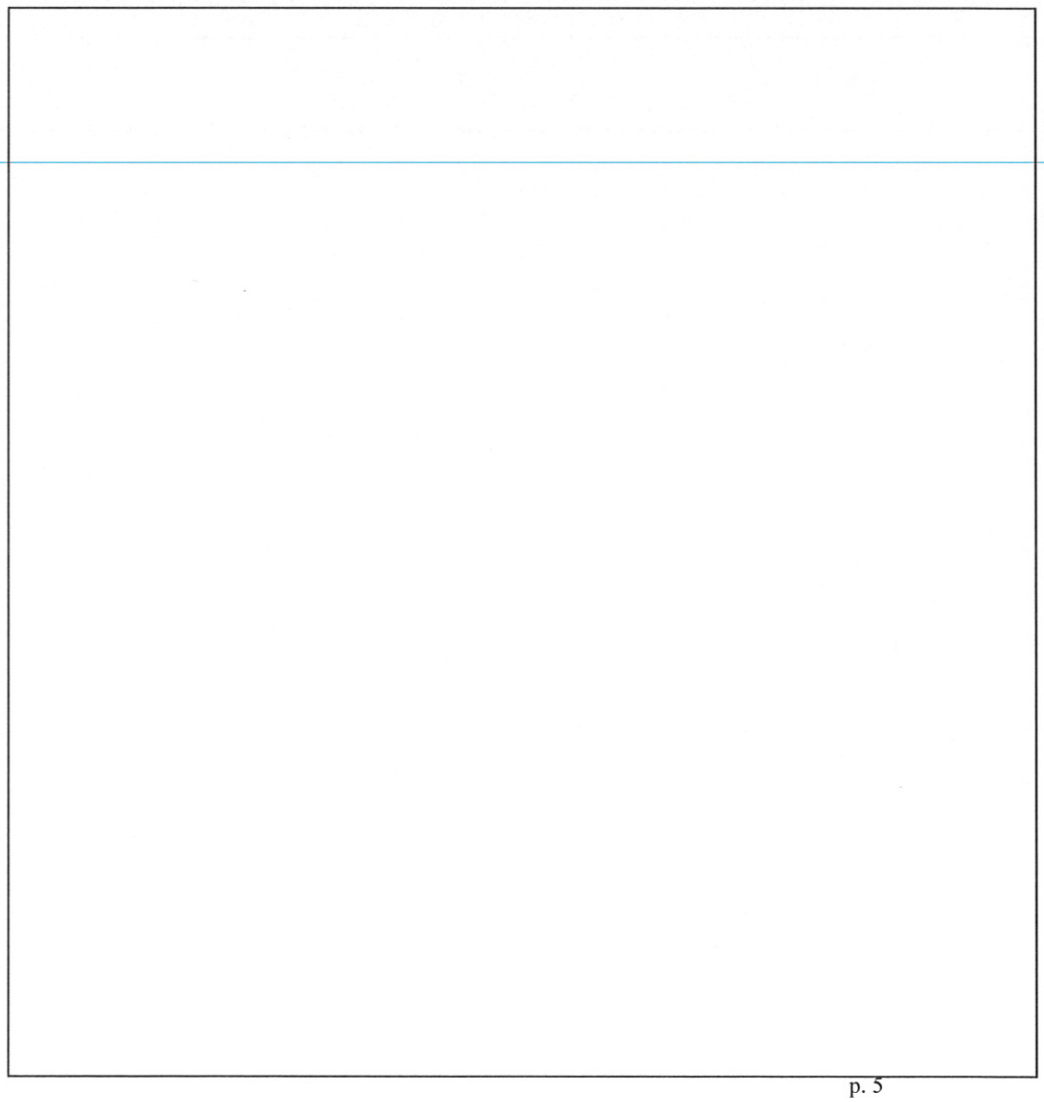
We have successfully deleted each of three key molecules (Sox2, $p27^{Kip1}$ and retinoblastoma protein (Rb)) in neonatal SCs within the organ of Corti and observed subsequent SC division; unfortunately, we did not find new sensory HCs in any of these models (Yu et al., *J. Neurosci*. 2010; Liu and Walters, et al., *J. Neurosci*., in press).

Given the importance of $p27^{Kip1}$ and its regulation in HC regeneration, we are employing fragment-based screening techniques to screen for inhibitors of $p27^{Kip1}$ that will allow cyclin dependent kinases to perform their catalytic activity during cell division. From the preliminary NMR (WaterLOGSY) screening and 2D ^{1}H-^{15}N HSQC verification, we have identified two primary hits that bind to a very important region of the kinase domain of $p27^{Kip1}$ (FYYR), which is responsible for inhibition of the ATP catalytic pocket of cyclin-dependent kinases. Using cheminformatics analysis, we have identified a third compound in the St. Jude

library that has a higher molecular weight, a similar core structure, and exhibits improved affinity for the kinase domain of $p27^{Kip1}$.These studies provide promising preliminary results for developing inhibitors of $p27^{Kip1}$ for HC regeneration.

Aim 2A: To create and characterize transgenic mice with inducible overexpression of Atoh1 in postnatal and adult SCs and measure the effects of Atoh1 overexpression on SCs after noise-induce HC damage.

When we acutely expressed Atoh1 in postnatal SCs using the newly generated transgenic mouse (CAG-loxP-stop-loxP-Atoh1-HA) and various CreER mouse lines, we observed SC-derived new sensory HCs in the postnatal mouse cochlea; however, pillar and Deiters' cell derived new HCs are immature (Liu et al., *J. Neurosci.*, 2012). Interestingly, new HCs derived from inner phalangeal cells (IPh) appear IHC-like with multiple HC markers, IHC morphology and nerve innervations, although the proteins, VGlut3 and prestin, are not expressed. Moreover, we analyzed the electrophysiological properties of these IPh-derived



new IHCs at various postnatal ages, and found that they exhibit expected potassium currents albeit smaller (**Fig. 4**). These results provide strong evidence that an Atoh1-mediated therapeutic approach may still be useful in regenerating new IHCs to restore some hearing, although these new IHCs are not fully mature and that similar to pillar cell and Deiters' cell-derived new HCs, other factors are needed in addition to Atoh1.

Aim 2B: To test the ability of γ-secretase inhibitors to increase Atoh1 expression in the mouse cochlea and measure their effects on HC morphology and hearing before and after noise-induced HC damage.

We have shown that reactivation of Notch signaling in the developing mouse cochlea resulted in ectopic HCs. However, such ability declines with age (Liu et al., *PLoS One*, 2012; Liu et al., *Dev Dyn.*, 2012). These results demonstrate that Notch manipulation alone is insufficient to induce HC regeneration in the adult cochlea. However, given our Atoh1 ectopic expression results, we are testing if the combination of Atoh1 activation and Notch manipulation would result in an enhanced amount of HC regeneration with more mature HCs than expression of Atoh1 alone. Furthermore, we have shown that Wnt signaling plays a key role in SC proliferation but not transdifferentiation (Chai and Kuo et al., *PNAS* 2012). Therefore, the combination of Wnt and Atoh1 is another promising avenue to regenerate HCs in the postnatal and adult cochlea. We will test the drugs proposed here (γ-secretase inhibitors) in combination with these *in vivo* genetic studies.

A. Work Plan

As described in **Expanded Accomplishments**, we will continue our exciting research as planned:

Aim 1A: To determine the regenerative capacity of SCs in $p16^{Ink4a}$-null mice after noise-induced HC damage. We will test the hypothesis that SCs are the cell source of the proliferative cells in the $p16^{Ink4a}$-null mice after NIHL.

Aim 1B: To transiently inactivate $p16^{Ink4a}$ after noise-induced damage. We will use the newly characterized Sox10-rtTA to manipulate p27, Sox2 and Rb in SCs to determine if transient inactivation of each results in proliferation and transdifferentiation of SCs into HCs.

Aim 1C: To develop small molecule inhibitors of $p27^{Kip1}$. We will continue our ongoing screen for $p27^{Kip1}$ inhibitors using fragment-based strategies and further design our secondary screening/verification strategies in HC/cochlear cultures.

Aim 2A: To create and characterize transgenic mice with inducible overexpression of Atoh1 in postnatal and adult SCs and measure the effects of Atoh1

overexpression after noise-induced HC damage. Given that Atoh1-induced IHCs from IPh cells exhibited electro-physiological properties albeit immature, we expect that after damage of endogenous HCs (either IHCs or OHCs or both), we should activate Atoh1 in IPh cells which should be converted into IHCs. Such mice should exhibit some levels of hearing. We are currently testing this exciting hypothesis; if proven in vivo, we will provide the first in vivo evidence of hearing restoration after HC damage, albeit small level, in mammals. However, damaging HCs in neonatal cochleae using Cre-independent methods is difficult and we are testing a few different approaches.

Aim 2B: To test the ability of γ-secretase inhibitors to increase Atoh1 expression in the mouse cochlea and measure their effects on HC morphology and hearing before and after noise-induced HC damage. We will test the combination of Atoh1-overexpression and manipulation of either Notch or Wnt signaling in vivo to achieve efficient HC regeneration.

B. Major Problems/Issues

Why don't mature cochleae regenerate HCs? Why do proliferation and transdifferentiation decline with age in vivo? These are the major and challenging questions that we are facing that will have to be overcome for HC regeneration and hearing restoration after NIHL.

We are grateful that we have received the ONR grant support to further identify and characterize factors and small molecules that would facilitate the maturation of OHCs and/or IHCs in conjunction with Atoh1 overexpression.

We are also happy that DURIP award from ONR has solved our previous rate-limiting problem with the confocal use time.

C. Technology Transfer

- We have contacted Sound Pharmaceuticals, Inc (Dr. J. Kil, another ONR awardee) on issues related to HC regeneration and ototoxicity in the past two years and will keep in touch on possible transfer of our mouse strains for their research. We also would like to share our results on p27 inhibitor screens with them for future development of drugs.

- We have visited CFD Research Corporation (Dr. Andrzej Przekwas, another ONR awardee) who is interested in future collaboration.

- We will interact with others to actively explore opportunities of transferring the new mouse strains and new small molecule inhibitors in the future.

D. Foreign Collaborations and Supported Foreign Nationals

We have hired several foreign nationals in our group as St. Jude employees using the ONR grant to work on this project.

- Dr. Luigi Iconaru, an experienced postdoc fellow with chemistry background and a Romanian national, is screening for small molecule inhibitors using NMR. He has made significant progress since he joined the lab in April 2010 and had since been awarded a prestigious postdoc fellowship award by St. Jude.
- Mr. Zhiyong Liu, an excellent PhD graduate student and a Chinese national, has been working on mouse models of Atoh1, p27 and Notch. He has made significant discoveries on all these areas and is a major contributor to the ONR funded project. He has graduated in Aug. and further stayed on continuing on his ongoing studies of HC regeneration until April 25, 2012. He is now a postdoc at the Howard Hughes Medical Institute at the Janelia Farm, Virginia.
- Ms. Lingli Zhang, a technician and a Chinese national, was hired in Aug. 2009 and has been working closely together with Dr. Cox on $p16^{Ink4a}$ and DTA and helping others for genotyping. She is an important member on all ONR funded projects.

No collaboration with foreign institutes/individuals.